After two hours at room temperature, 10 ml of the fixed hyphoe were centrifuged at 3000 rpm for 10 min, the hyphoe were resuspended in 10 ml of PB and recovered in buffer complete. The washed, fixed hyphoe were then resuspended in 10 ml PB containing 0.2 μg/ml of DAPI and left for 15-16 hours at 4°C. Although the dilute DAPI solution is unstable, a stock solution of 1 mg/ml in distilled water can be stored for weeks at -20°C. After staining, the hyphoe were recovered by centrifugation, washed twice with PB, resuspended in a small volume of buffer and mounted. Observations were made with an optical fluorescence microscope (Leitz Ortholux equipped with 1 mm UG-1 excitor filter, 5 mm BG-38 red absorbing filter and a barrier filter K.430 or K.460). Nuclei appear as bright, light-blue spherical bodies, while the cytoplasm is almost completely dark, except for small foci of fluorescence which are probably due to mitochondrial DNA. Septa are also visible as weak dark-blue linier.

With a suitable microfluorimeter (we employed a Leitz-MPV microphotofluorimeter with a KNOTT-MFLK photoelectric unit) the intensity of the fluorescence of individual nuclei can be measured, and the relative DNA content of each nucleus con therefore be determined. The staining with DAPI appears very stable under UV light, with no appreciable fading.

Perlman, J. and J.F. Feldman.

A new culture method for biochemical study of the circadian clock.

The band strain of N. crassa exhibits a well-characterized circadian rhythm of conidiation under constant conditions of temperature and darkness (Sargent et al., 1966 Plant Physiol. 41: 1343-1349). Biochemical and physiological study of the circadian clock have been very difficult because most cultures used in such experiments have been grown on solid medium to observe the bonding pattern. We have now been able to show that homogeneous, non-bonding cultures also exhibit normal circadian rhythmicity included in our studies were lawn-inoculated cultures on solid medium, shaking liquid cultures, and standing liquid cultures. Methods and results are presented only for the latter because the system has been characterized the most extensively and potentially may be of the most use.

Cultures were maintained on Horowitz complete slants. All other medium, both liquid and solid, was Vogel's salts containing 1.2% sodium acetate and 0.05% casamino acids (solid medium contained 1.5% agar). A conidium from a 6-8 day old slant was suspended in distilled water or (in later experiments) liquid medium, and filtered through glass wool. The concentration of conidia in the filtrate was measured (Klett-Summerson colorimeter, blue filter), and an aliquot immediately added to a large volume of stirring liquid medium to give a final concentration of 2x10⁵ conidia/ml. Using an automatic pipette, 25 ml of the stirring suspension were added to each of several dozen 100 x 15 mm plastic disposable Petri dishes. Six growth tubes with solid medium were inoculated at one end with about 50 microliters of undiluted filtrate. All plates and growth tubes were put in constant light at 25°C. After about a day, they were transferred to constant darkness in an environmental growth chamber, also at 25°C. The Petri dish cultures had visible growth by 24 hours, and subsequently formed a mycelial mat which covered the surface. At several different times after the cultures were placed in the dark, six pieces of mycelium from each of three plates were cut with a cork borer (11 mm diam.) and transferred to fresh growth tubes. (The size of the transferred piece has been varied greatly with identical results.) The growing fronts of control and experimental growth tubes were marked in red safelight (G.E. BCJ, 60 watt) at the same time each subsequent day. After about 7 days in the dark, the timer of occurrence, or phases, of the first conidial bands of the experimental growth tubes were determined by linear regression analysis and compared to the corresponding band of the control tube.

The phases observed in the growth tubes inoculated with pieces of the standing liquid cultures were very close to those of the controls at all timer sampled. (In some experiments small and consistent phase advances were seen in the experimental tubes.) The sampling manipulations therefore do not affect the phase of the clock, and it may be concluded that the liquid cultures have a normal circadian clock whose phase is set, like that of the controls, by the light-to-dark transition. The phases of experimental growth tubes tend to differ from those of the controls of approximately the time when the liquid cultures reach stationary phase (about 55-60 hours of age). It seems likely that either the clock of older cultures "runs down," or that older cultures are susceptible to phase resetting when cut and transferred. This appears to be largely independent of the length of time they spend in constant light. Preliminary experiments suggest that a 3 hour period in constant light (possibly even less) suffices to set the phase of these cultures.

Experiments are underway to relate the age of the cultures and nuclear division timer to the functioning of the clock. Other types of experiments, particularly the addition of various agents to determine their effects on the clock, are now possible.

Rigby, D.J., T.J. Balls and A. Radford.

Semi-quantitative analysis of protease activity.

A technique has been developed in this laboratory by which Nematcara proteases can be quickly and simply assayed semiquantitatively. The method is particularly useful when many samplers must be assayed. The assay is based on the digestion of the gelatin matrix of the emulsion of photographic film, digestion progressively
releasing the bound silver groins until only the transparent backing of the film remains. The time required for complete digestion, and hence complete clearing, was found to be proportional to the protease concentration.

In our standard method, fractions to be assayed (0.4 ml) were dispensed into 10 x 75 mm tubes, and the temperature equilibrated to 37°C. Into each of the tubes was placed a 2.5 x 35 mm strip of exposed photographic film, and the time required for complete clearing of that part of the film below the surface was determined.

The assay was calibrated with trypsin (Wellcome Laboratories), diluted when desired with 0.05 M phosphate buffer, pH 7.3. A wide range of trypsin concentrations were studied (Fig. 1). A linear plot was obtained with 0.3 to 1.25 x 10^-3% of trypsin, but outside this range accurate determination of the end point was not possible. Even so, trypsin concentrations of 3 x 10^-4% and even lower were detectable by this method.

![Figure 1](image1.png)

![Figure 2](image2.png)

Using the above method, the distribution of protease activity in a Sephacryl S-200 eluent (column size 85 x 2.5 cm, 5 ml fractions) of a crude extract of Neurospora mycelium was investigated. The results are shown in Figure 2. The proteases were eluted continuously from the column, suggesting that they were bound to molecules of much higher molecular weight (presumably their substrates), and dissociate continuously to yield free protease molecules, separated by the effects of gel filtration. The large peak of protease activity corresponds to the high molecular weight peak of aggregated proteins. (Supported by S.R.C. Grant GR/A/64655.)

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Tublitz, N., D. Foster and P. J. Russell. Isolation of ribosomes from Neurospora and their analysis using a vertical rotor.

Differential ultracentrifugation has enabled researchers to isolate and purify ribosomes. Conventional isopycnic centrifugation techniques to resolve ribosomal subunits have used swinging bucket rotors requiring long, time-consuming spins. This paper reports a new technique for ribosomal subunit separation in a sucrose density gradient by using a vertical rotor. We also report the effect of various storage conditions on the stability of Neurospora crassa ribosomes.

The Dupont Sorvall TV850 vertical rotor contains eight fixed, vertically-positioned tube apertures. During controlled, slow acceleration, tube contents are reoriented 90°, producing a very narrow sample zone and increasing the slope of the gradient. Re-orientation also significantly decreases run time due to shortening of the path length through which the sample must travel.